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In vitro activation of human herpesviruses 6 and 7 from latency

(exanthem subitum/transplantation/bone marrow transplant)

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ABSTRACT **Human herpesviruses 6 and 7 (HHV-6 and HHV-7) are prevalent lymphotropic viruses that infect more than 80% of children at infancy or during early childhood. Infection ranges from asymptomatic to severe disease. HHV-6B causes exanthem subitum. The virus can be recovered from peripheral blood mononuclear cells during the acute phase of exanthem subitum, but the host remains latently infected throughout life. In immunocompromised patients undergoing kidney, liver, or bone marrow transplantation latent HHV-6B is reactivated, at times causing severe or fatal disease. Here, we describe the establishment of an *in vitro* system for reactivation of HHV-6B and HHV-7 from latency. HHV-7 is reactivated from latently infected peripheral blood mononuclear cells by T-cell activation. HHV-6B could not be reactivated under similar conditions; however, the latent HHV-6B could be recovered after the cells were infected with HHV-7. Once reactivated, the HHV-6B genomes became prominent and the HHV-7 disappeared. We conclude that HHV-7 can provide a transacting function(s) mediating HHV-6 reactivating from latency. Understanding the activation process is critical for the development of treatments to control the activation of latent viruses so as to avoid these sometimes life threatening infections in transplant recipients.**

Human herpesvirus 6 (HHV-6) was initially recognized in peripheral blood mononuclear cells (PBMCs) of patients with lymphoproliferative disorders (1). Soon after the isolation of the first HHV-6 strain (GS), additional HHV-6 isolates were obtained, including the U1102 and Z29 strains, which were isolated from AIDS patients in Uganda and Zaire, respectively (2, 3). Numerous isolates of HHV-6 obtained thereafter fell into two groups of viruses differing in growth properties, antigenicity, location of restriction enzyme sites, and DNA sequences, as well as epidemiology (4–11). The two types of HHV-6 isolates were designated HHV-6A and HHV-6B variants (12).

Human herpesvirus 7 (HHV-7) was first isolated from $CD4^+$ T-cells of a 25-year-old healthy individual (RK), most likely following its reactivation from latency (13). It was later shown that HHV-7 is present in the saliva of healthy individuals (14–16). The HHV-7 strains were distinct from HHV-6A and HHV-6B variants, as shown by restriction enzyme blot hybridization analyses, nucleotide sequencing, and interactions with monoclonal antibodies. Diagnostically they can be distinguished by immunofluorescence assays and PCR (10, 16–19). HHV-6 and HHV-7 are prevalent viruses infecting more than 80% of healthy children (reviewed in refs. 11, 16, and 20).

In 1988, Yamanishi and coworkers (21) reported that HHV-6 is the causative agent of exanthem subitum (ES or roseola infantum). ES is a common disease of infants or young children characterized by 3–4 days of high fever, followed by skin rash (11, 20, 21). Restriction enzyme analyses of DNAs of isolates obtained from peripheral blood of ES patients showed that these viruses were HHV-6B variants, similar to the Z29 prototype strain (6). In some cases ES is accompanied by a wide range of clinical complications from meningoencephalitis to fatal fulminant hepatitis (11, 20, 22, 23). It was suggested that HHV-7 also causes ES, although in many of these cases the observed ES was associated with a large increase of HHV-6 antibody titers (16, 19, 24, 25). We hypothesize that the HHV-7 primary infection was coupled to reactivation of HHV-6B from latency leading to the ES symptoms.

HHV-6 can be reactivated from latency in patients undergoing immunosuppressive treatments prior to kidney or liver transplantation, as well as patients undergoing bone marrow transplantation (BMT) (9, 11, 26–32). It has been suggested that HHV-6 infection can cause failure of the transplant (29). Furthermore, in some cases HHV-6 infection caused life-threatening interstitial pneumonitis and encephalitis (28–30). Analysis of viruses recovered during renal or pediatric allogeneic transplantation revealed that they were HHV-6 type B strains (9). HHV-6 reactivation in the allogeneic BMT patients was associated with long-term (weeks) viremia (9), unlike ES in immunocompetent children, where viremia lasts only 3–4 days (20–25).

In this paper we describe the establishment of an *in vitro* system for reactivation of HHV-6B and HHV-7. We show that HHV-7 can be induced from latency in PBMC by T-cell activation, whereas HHV-6 cannot be similarly reactivated. HHV-6B variants are reactivated following infection of the PBMC with HHV-7, suggesting virus-helper interactions. The consequences of viral reactivation *in vivo* are discussed.

MATERIALS AND METHODS

Cells and Viruses. Freshly prepared PBMCs of healthy individuals were fractionated in lymphocyte separation medium, as described (33). Aliquots of the isolated PBMC were frozen at −80°C. The cells were preactivated in RPMI 1640 medium containing 5 μg/ml gentamicin, 10% fetal calf serum, and 10 μg/ml phytohemagglutinin (PHA) as described (33) except that no interleukin 2 (IL-2) was added to infection media in these experiments.

To determine induction from latency, the cells were activated with PHA for 3 days, spun, and resuspended in media without PHA. The cells were incubated for 1 week, then mixed with additional preactivated PBMCs from the same donor or from different donors as detailed in the *Results*. Cells were mixed at a ratio of 1 part old PBMC to 9 parts new PBMC. Viral replication was first noted by the appearance of a

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Abbreviations: HHV, human herpesvirus; ES, exanthem subitum; BMT, bone marrow transplantation; PBMC, peripheral blood mononuclear cell; MLR, mixed lymphocyte reaction; PHA, phytohemagglutinin; IL-2, interleukin 2.

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cytopathic effect. The resultant virus was identified by immunofluorescence assays and DNA analyses.

To test the effect of HHV-7 on HHV-6 induction, the cells were treated with PHA and then infected with HHV-7 RK strains. The resultant virus was passaged through additional pre-activated PBMCs. Controls were preactivated and mock infected in an identical manner.

Immunofluorescence Assays. Cells were fixed with acetone and then reacted with monoclonal antibodies derived by Bala Chandran (University of Kansas Medical Center) against HHV-6A strain GS (34). The slides were stained with fluorescein isothiocyanate-conjugated rabbit and mouse immunoglobulins. Some of these monoclonal antibodies crossreacted with the HHV-6B variant or with HHV-7 as described (11, 14, 17).

DNA Analyses. Virus and strain identity were determined by analyses of restriction enzyme polymorphism as described (6, 33).

RESULTS

Infection of PBMCs with HHV-7 Results in the Activation of Latent HHV-6 Genomes. In the course of initial studies of HHV-7 in the laboratory, propagation of HHV-7 (RK) in PBMCs sometimes yielded mixed infections comprised of both replicated input HHV-7 and new HHV-6 isolates. Further virus propagation in PBMCs resulted in enrichment of the generated HHV-6. Because the generated HHV-6 did not arise from the HHV-7 inoculum, it seemed likely that the new HHV-6B strains were induced from latency. Because new HHV-6 was not generated in mock infections, it seemed likely that the replicating HHV-7 was assisting the reactivation of HHV-6. Fig. 1 schematizes attempts to reproduce HHV-6 reactivation during serial propagation of HHV-7 in PBMC cultures. Three preparations of HHV-7 were employed: 7/RK is a generalized virus stock prepared in cord blood. RK1 and RK2 designate the initial virus preparations in the $CD4^+$ T-cells isolated from RK. The T-cells had been activated with anti-CD3 and IL-2 (RK1) or anti-CD3 and anti-CD28 monoclonal antibodies (RK2) (13) to recover HHV-7. These stocks were used to infect PBMCs from different donors in series. Individual PBMCs were denoted by the numbers in Fig. 1. The serial propagation was performed "blindly," allowing 1 week for each passage. The propagated viruses were examined by immunofluorescence assays and by restriction enzyme analyses of viral DNAs.

The results can be summarized as follows: (*i*) In some cases serial infection of PBMCs with HHV-7 yielded a virus that resembled the input HHV-7 (e.g., Fig. 1*A*, PBMC 1–6). (*ii*) In other cases, propagation of HHV-7 in the PBMCs yielded mixed HHV-6 and HHV-7 stocks (e.g., PBMC 7). (*iii*) The emerging HHV-6 overtook the virus population in the series (PBMC 8), indicating that HHV-6 replicates more efficiently than the input HHV-7 strain under these conditions. The emerging HHV-6 isolates were designated as strains MG1–MG7. (*iv*) The generated isolates were grossly similar to HHV-6B (Z29), but they could be distinguished on the basis of restriction enzyme polymorphism (Fig. 2). (*v*) HHV-7 strain RK, which replicated in PBMC 1, was serially propagated in two series: PBMC 2–8 and PBMC 12–18 (Fig. 1*A*). This propagation resulted in the emergence of HHV-6 strains MG1 and MG2, respectively. Thus, different strains of HHV-6B were activated in PBMC preparations from different donors using the same helper HHV-7 strain. (*vi*) Propagation of the HHV-7 (RK1) and (RK2) through the same PBMCs (5–8, 31–33) yielded in both cases HHV-6 strain MG4 recoverable by the seventh passage (Fig. 1*C*). Both isolates were considered to represent the same strain MG4, because their restriction fragment patterns were identical for at least six enzymes. We hypothesize that the HHV-6B strain MG4 was activated

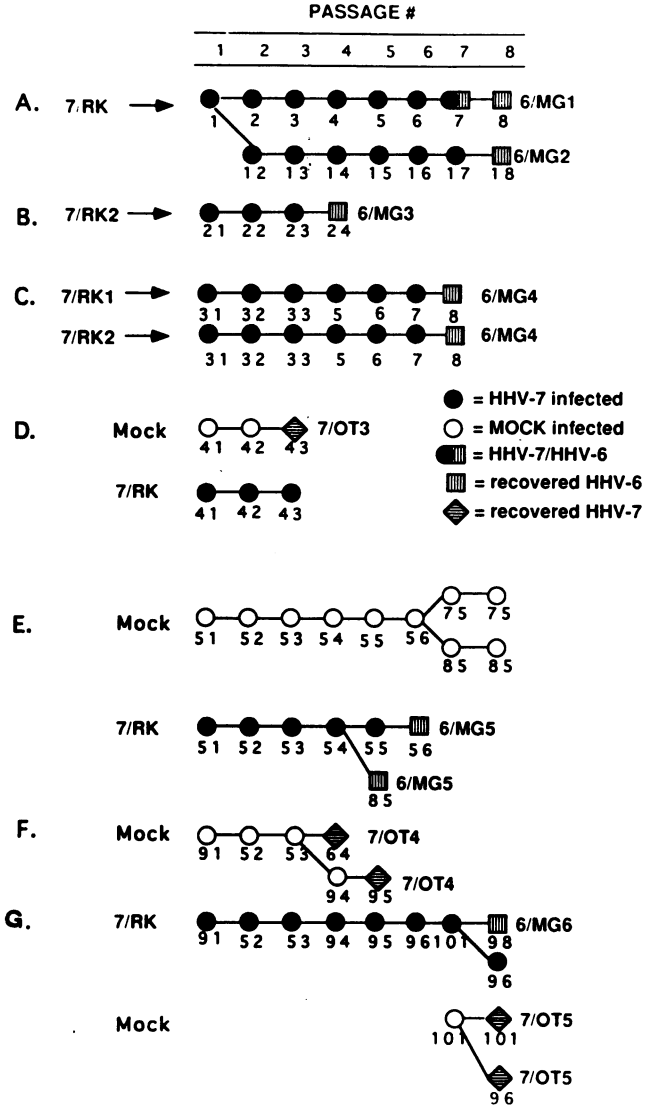

FIG. 1. *In vitro* activation of HHV-6 and HHV-7. Propagation of HHV-7 in PBMCs resulted in the activation of latently infected HHV-6. MG1-MG6 are different HHV-6 isolates. OT1-OT5 are variants of HHV-7 isolated by activation and mixing of uninfected PBMCs. (*A*) Propagation of the same HHV-7 isolate in different PBMCs results in the activation of two distinct isolates of HHV-6. (*B*) The number of passages required to see reactivation of latent HHV-6 is variable. (*C*) Two different HHV-7 isolates propagated through the same set of PBMCs result in activation of the same HHV-6 variant. (*D–G*) Identical sets of PBMCs were infected with HHV-7 (7/RK) or were mock infected. (*D*) Activation of PBMCs without HHV-7 infection results in activation of HHV-7 from latency. (*E*) HHV-7 was not always activated in mock cultures. (*F* and *G*) When one PBMC was used as the inoculum for two different PBMCs, HHV-6 was activated in only one of the two 7/RK-infected PBMCs (*F*), but the same HHV-7 strain appears in both PBMCs in the next passage for mock-infected cells (*G*).

independently during passaging of different HHV-7 preparations in the same series of PBMCs. (*vii*) Activation and recovery of HHV-6B from a latent/quiescent state did not depend on the number of different PBMC preparations through which the HHV-7 was propagated. HHV-6B strains MG1, MG2, MG4, and MG6 were recovered after seven or eight passages of the HHV-7 "helper" virus in different PBMC preparations, while strain MG3 emerged in the fourth passage (Fig. 1*B*). We conclude that infection of PBMCs with HHV-7 can yield HHV-6B variants, reactivated from latency in different PBMCs. The DNA polymorphism in the rescued MG-

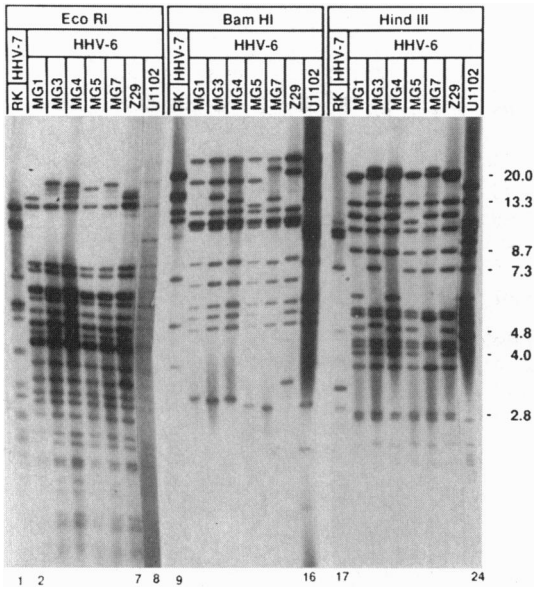

FIG. 2. HHV-6 strains recovered from latency. MG1–MG7 are different strains rescued from latency by passaging HHV-7 RK in different PBMCs. Viral DNA from each new HHV-6 isolate was labeled with ^{32}P-orthophosphate during infection, recovered, cleaved with the restriction endonucleases listed, and subjected to electrophoresis and fluorography. Note that all of these strains are similar to HHV-6B (Z29) and different from HHV-6A (U1102) or HHV-7 (RK). Also note that there is hardly any HHV-7 left (i.e., following activation, HHV-7 is competed out by the newly activated HHV-6).

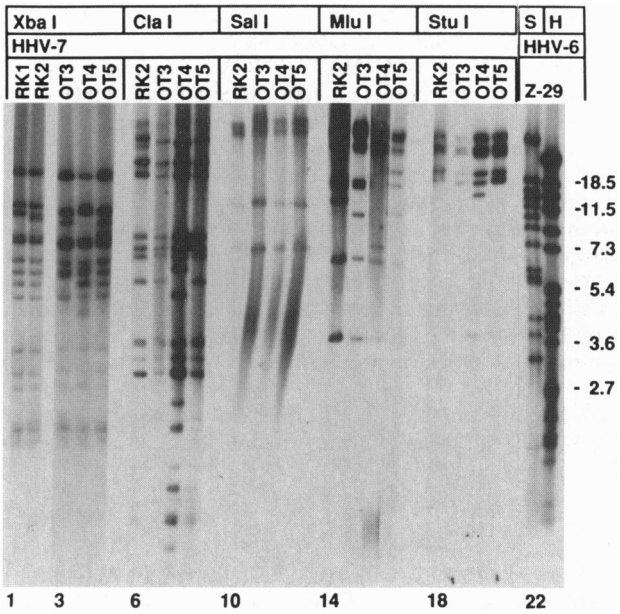

FIG. 3. Recovery of HHV-7 strains. RK1 and RK2 are HHV-7 strains arising following activation of CD4$^+$ T-cells with anti-CD3 and IL-2 or anti-CD3 and anti-CD28, respectively. Strains OT3, OT4, and OT5 were activated following PHA treatment of the PBMCs and mock passage of PBMCs to elicit the mixed lymphocyte reaction (MLR). DNAs were recovered as in Fig. 2. S and H indicate *Sal*I and *Hin*dIII, respectively.

type strains resembled the heterogeneity seen in virus pools recovered from BMT patients (9) and from ES patients (6). Once generated, the MG-type viruses were homogeneous and stable. They did not change upon further propagation (data not shown).

Reactivation of HHV-7 from Latency. Fig. 1 *D–F* chronicles mock-infected control PBMCs, which were included in parallel to the HHV-7-infected PBMC cultures. Two days prior to infection or mock infection, the cultures were exposed to PHA so as to induce T-cell activation. The results can be summarized as follows: (*i*) As expected, exposure of the mononuclear cells to PHA resulted in T-cell activation, as judged by the size of the cells, and an increase in cell numbers. (*ii*) PHA treatment of some of the mock-infected cultures resulted in cytopathic effect. The viruses recovered were all HHV-7 strains (example restriction enzyme patterns, Fig. 3). (*iii*) Variability in the restriction enzyme patterns was similar to the heterogeneity of HHV-7 strains present in saliva samples of healthy individuals (14, 15) or in HHV-6B strains in BMT patients (9). The HHV-7 strains recovered in this experiment were designated OT3, OT4, and OT5 (Figs. 1 *D*, *F*, and *G* and 3). (*iv*) The reactivation of HHV-7 did not depend on the number of virus passages. OT3 was recovered in the third passage, OT4 in the fourth and fifth passages, and OT5 in the second passage. Mock infection and propagation of another batch of PBMCs did not result in apparent virus up to passage 8, which was the last passage tested (Fig. 1*E*). (*v*) In a paradigm where mock-inoculum PBMC 101 was mixed with another aliquot of PBMC 101 an HHV-7 isolate emerged, most likely resulting from amplification of a reactivated HHV-7. Similarly, propagation of a PBMC 101 aliquot with PBMC 96 resulted in the appearance of HHV-7. Restriction enzyme analyses with more than six enzymes revealed the identity of the two isolates. The emerging virus most likely resulted from activation from latency followed by virus replication increasing the amount of rescued virus. The virus strain emerging from PBMC 101 was designated OT5 (Fig. 1*G*). It is noteworthy that the original isolation of HHV-7 strain RK also followed T-cell activation upon exposure of the cells to anti-CD3 plus IL-2 (RK1) or anti-CD3 plus anti-CD28 (RK2) (13). None of the mock-infected PBMCs yielded HHV-6 variants without being first infected with HHV-7. Thus, reactivation of HHV-7 from latency required T-cell activation, whereas the reactivation of HHV-6B from latency required a helper virus.

HHV-6 Reactivation Is Affected by the Mixed Lymphocyte Reaction (MLR). An experiment was set up to test whether MLR plays a role in viral reactivation. The effect of mixing PBMCs from different individuals might be of clinical relevance with regards to allogeneic BMT, bringing about MLR. An experiment was set up to test the effect of PBMC mixing. Four different PBMC cultures (75, 76, 85, and 99) were prepared and frozen in small aliquots. This allowed continuous viral propagation in given PBMCs, using for each passage another frozen aliquot of the same PBMC. Infections were carried out employing the same PBMC repeatedly or employing particular combinations of PBMCs. Mock infections were carried out in the same PBMC combinations as the HHV-7 (RK) infections. Fig. 4 summarizes the set up and results of the infections. Fig. 5 (lanes 1–13) display the restriction patterns of ^{32}P-labeled DNAs from passages four of all the series, whereas lanes 14–17 exemplify the passages in a single series. The data revealed the following: (*i*) Most passages contained replicated input HHV-7 DNA. (*ii*) In some cases, the HHV-7-infected cells yielded rescued HHV-6B isolates. For example, passage 5 of the induced virus contained mostly the rescued HHV-6B with almost no residual HHV-7 DNA (Fig. 5, lanes 17 and 18). (*iii*) Mock-infected cultures for all series were propagated through passage four. No cytopathic effect was noted in any of the mock-infected cultures, and no viruses were recovered from these preparations (data not shown). (*iv*) As seen in Fig. 5, the cells that were infected with HHV-7 and passaged using the same frozen PBMCs, replicated virus similar to the input HHV-7 (RK). (*v*) Mixing HHV-7-infected PBMC 75 and 85 (lanes 5 and 8) led to the recovery of an HHV-6B isolate. (*vi*) The virus induced in the 75 into 85 series was identical to the virus induced in the 85 to 75 series. The viruses were designated MG7. Passage alternating between 85 and 99 yielded

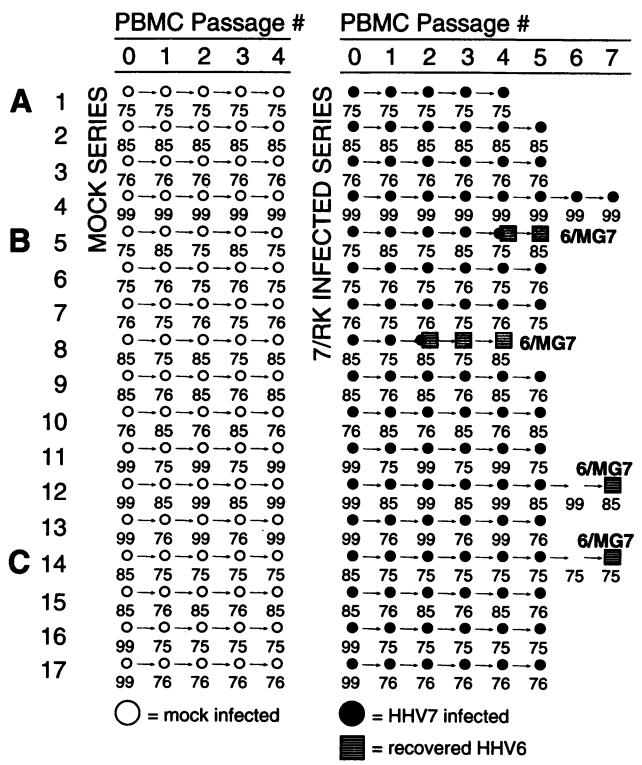

FIG. 4. MLR affects the activation of HHV-6. Propagation of HHV-7 in activated PBMCs in combination with MLR results in the activation of latently infected HHV-6 viruses. The experimental protocol and results are diagrammed with different PBMCs given the patterns indicated in the legend. (*A*) When continuously propagated in the same PBMCs without MLR, HHV-7 infection failed to activate HHV-6 from latency. (*B*) Propagation of HHV-7 in alternating PBMCs resulted in rapid activation of HHV-6 from latency. (*C*) Propagation of HHV-7 with one MLR followed by continuous passaging resulted in activation of latent HHV-6 after seven passages.

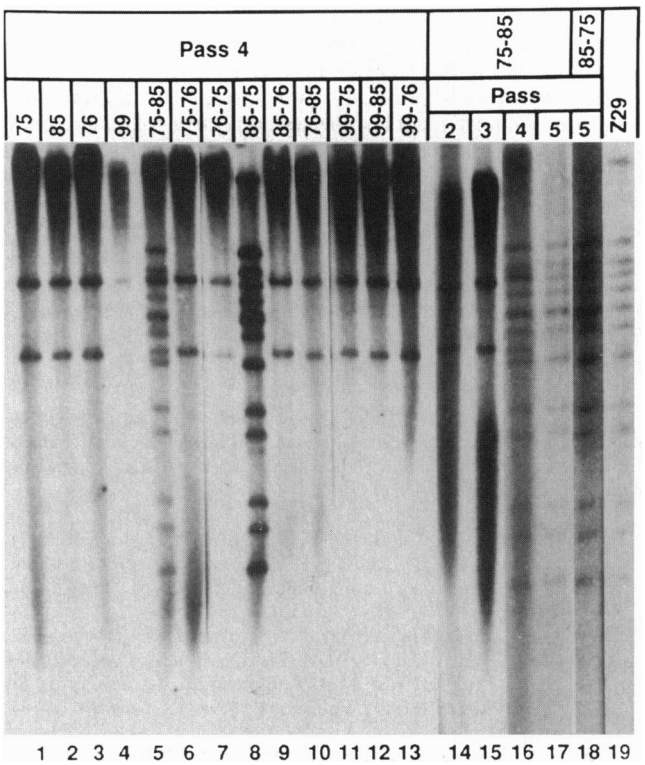

FIG. 5. Analysis of the experiment schematized in Fig. 4. DNA analysis of passage four from the experiment schematized in Fig. 4. Viral DNAs were prepared as in Fig. 2. Lanes 1–4 correspond to lanes 1–4 in Fig. 4, whereas lanes 5–13 correspond to lanes 5–13 in Fig. 4. Lanes 14–17 follow the 75–85 series across several different passages. Lane 18 compares the recovered virus from two different series in passage five.

HHV-6 by the seventh passage. The virus was also indistinguishable from MG7. It appeared that the MG7 strain was activated from PBMC 85, in response to HHV-7 infection because the HHV-6 isolates had identical restriction fragment patterns and PBMC 85 was the only PBMC used in all series from which HHV-6 was isolated. (*vii*) Passaging 85 then 76 did not yield an HHV-6 isolate at least until passage five, the last passage to be tested. It can thus be concluded that the cross interactions with some PBMCs are prone to virus reactivation. Furthermore, serial passaging of HHV-7 in the PBMC 85 aliquots did not result in activation; so it would seem that either infection with HHV-7 alone is not sufficient to activate latent HHV-6 genomes or that the MLR speeds up the process of activation. Because the experiment was terminated after passage seven, we do not know if infection with HHV-7 alone could eventually result in activation of the latent HHV-6 genomes, but clearly the MLR speeds up the process of activation if it is not required for the activation. We conclude that the HHV-6 can be reactivated from latency in response to HHV-7 infection. Furthermore, MLR plays a role in this virus reactivation.

DISCUSSION

The molecular basis underlying the transitions between lytic virus replication and latency are as yet poorly understood. To date, latency in HHV-6 or HHV-7 is merely defined operationally: the virus does not replicate during latency in quiescent cells; however, it retains the capacity to be reactivated.

HHV-7 was first isolated through activation from latency in purified $CD4^+$ T-cells of a healthy individual (13). However, the range of other cell types in which HHV-7 can establish latency has not been investigated. Filterable cell-free HHV-7 is persistently present in human saliva of more than 75% of healthy individuals (14, 15). The virus replicates well in $CD4^+$ cells from PBMC or cord blood, but, it is as yet unknown which cell type(s) contains the replicating virus in salivary glands. Understanding why HHV-7 replicates continuously in one cell type, but establishes latency in another, will be useful to understand viral tropism and latency. As recently shown by Lusso and colleagues (35), the CD4 constitutes at least a portion of the receptor for HHV-7. Furthermore, the virus can replicate in SupT1 human T-cell lines (ref. 35 and D. Rapaport and N.F., unpublished observations).

Here we have established an *in vitro* system in which we can observe latency and reactivation of HHV-7 and HHV-6. We have shown that PHA activation of PBMCs combined with the mixed lymphocyte response result in activation of latent HHV-7. This is a practical system to study viral reactivation from latency. HHV-6 replication depends on T-cell activation (36). However, unlike HHV-7, T-cell activation is insufficient for HHV-6 reactivation from latency. Activation of HHV-6 from latency requires other factor(s). We have shown that HHV-6 can be induced *in vitro* by infecting the cells with HHV-7. This mechanism could also apply *in vivo*: it was reported that HHV-7 can also cause ES, but in the majority of these patients higher titers against HHV-6 appeared as well, reflecting HHV-6 reactivation in the presence of primary HHV-7 infection (19). Furthermore, in some transplant patients, both HHV-6 and human cytomegalovirus have been isolated. HHV-6 antibody titers rise in some transplant patients with primary human cytomegalovirus infection (27, 37).

The consequences of viral reactivation range from asymptomatic to fatal interstitial pneumonitis and encephalitis in patients undergoing BMT (28, 29). Moreover, HHV-6 infections are associated with morbidity and mortality in the immunocompromised host, most notably in kidney transplant and bone marrow transplant patients. The time for virus reactivation appears variable. HHV-6 viremia, at times accompanied by skin rash, has been reported 2–4 weeks after transplantation. Studies of HHV-6 reactivation from latency are clinically important. They might lead to prevention of, or earlier detection of, viral reactivation *in vivo*. This opens the door to potential treatments with anti-viral substances for short duration in cases where these potential antiviral drugs cannot be employed as prophylactic treatment because of toxicity.

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